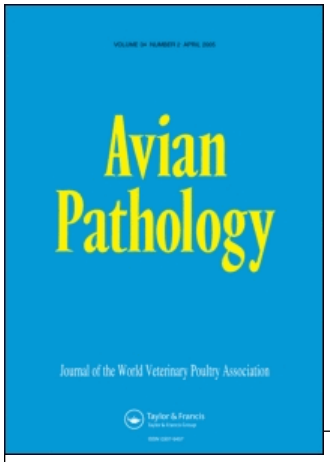

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The VG/GA strain of Newcastle disease virus: mucosal immunity, protection against lethal challenge and molecular analysis

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The VG/GA strain of Newcastle disease virus: mucosal immunity, protection against lethal challenge and molecular analysis

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The Villegas–Glisson/University of Georgia (VG/GA) strain of Newcastle disease virus (NDV) isolated from the intestine of healthy turkeys has been proposed to replicate in the respiratory and intestinal tract of chickens. In the present study, the virus distribution, the mucosal and systemic immune response, the efficacy against lethal challenge and the full genome sequence of the VG/GA strain were compared with the La Sota strain of NDV. The VG/GA strain was detected at different time points in the respiratory and intestinal tract of chickens with a preferential tropism for the latter. Both the VG/GA and La Sota strains induced NDV-specific immunoglobulin A (IgA) at the upper respiratory tract. IgA levels were higher in the trachea for the La Sota strain, while they were higher in the bile and intestine for the VG/GA strain. Positive correlation between virus distribution of the viruses and IgA production was observed. Despite the presence of the maternal antibodies in broilers, early vaccination with the VG/GA strain afforded 95% to 100% protection against lethal challenge, equivalent to the protection conferred by the La Sota strain. Full genome sequence analysis classified the VG/GA strain within class II, genotype II viruses, which also include most of the respirotropic vaccine strains. Differences with the La Sota strain at the nucleotide and amino acid levels that may explain the differential phenotype of the VG/GA were observed; however, verification of the significance of those changes is required. Taken together, these results validate field observations on the efficacy of VG/GA vaccination and demonstrated the unique characteristics of the strain.

Introduction

Newcastle disease virus (NDV) is one of the most important infectious agents in the poultry industry, affecting a wide variety of birds and causing important economical losses (Alexander, 2001). The virus belongs to the family *Paramyxoviridae*, subfamily *Paramyxovirinae*, in the genus *Avulavirus* (Mayo, 2002). Vaccination of commercially reared birds has been considered the best way to reduce losses resulting from NDV infection (Senne *et al.*, 2004). For primary NDV vaccination, the vaccine of choice is one that elicits adequate immune response with minimal respiratory reactions (Villegas, 1998).

The Villegas–Glisson/University of Georgia (VG/GA) strain of NDV has been proposed to replicate both in the respiratory and intestinal tract, with preference for the intestine. Vaccine-induced respiratory reactions are decreased or avoided when using the VG/GA strain, probably by diminishing the level of replication in the respiratory epithelia of young chickens (Nunes *et al.*, 2002). Initial investigations with the VG/GA strain stated that the virus produced no detectable respiratory reaction in chickens regardless of the vaccination route used, it afforded respiratory tract protection against challenge with a respiratory-type NDV (La Sota strain) and it produced high haemagglutination inhibition titres showing no interference with immunity to infectious bronchitis (Glisson *et al.*, 1990).

Most of the commercially available lentogenic vaccines are able to induce antibodies against NDV; however, systemic humoral immune response measured as the presence of specific NDV antibodies in serum is not enough for protection (Erf, 2004; Reynolds & Maraqa, 2000). It has been established that the mucosal immunity represented by immunoglobulin A (IgA) production plays an important role in the development of protection in chickens vaccinated against Newcastle disease (Reynolds & Maraqa, 2000; Scott, 2004; Seal *et al.*, 2000). Antibody production in the mucosa is closely related to viral replication in the target cells, hence the pathogenesis and tissue tropism of the viruses used for vaccination is to be considered in order to assess the efficacy of a given live vaccine against a direct challenge (Jayawardane & Spradbrow, 1995). The intestinal tropism of the VG/GA strain and the consequent induction of local immunity may be important for protection against velogenic-viscerotropic strains of NDV that have been reported to induce massive destruction of intestinal lymphoid areas and extensive

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ulceration of overlying intestinal epithelium (Brown *et al.*, 1999).

The VG/GA strain, when applied to immune-competent specific pathogen free (SPF) chickens, induces protection against lethal NDV challenge (Beard *et al.*, 1993). Also, anecdotal data obtained from field experiences indicate that the VG/GA strain is useful in the control of velogenic-viscerotropic NDV strains in poultry. Full protection against lethal challenge has been reported when the VG/GA strain is included in vaccination programmes under experimental and field conditions (Perozo *et al.*, 2004; Silva *et al.*, 2004).

The reason the VG/GA and some asymptomatic enteric strains preferentially target intestinal epithelia cells is unknown. Changes in the structural proteins like the polymerase protein (L), the fusion (F) protein or the haemagglutinin-neuraminidase (HN) protein have been reported recently as major determinants of tropism and virulence (de Leeuw *et al.*, 2005; Huang *et al.*, 2004) and could explain the differential tissue tropism observed among lentogenic strains. The HN and F proteins may determine a unique attachment and fusion phenotype that allows the enterotropic viruses to enter the intestinal cells preferentially and the differences in the L protein may allow higher levels of replication for the enterotropic strains or impair the replication of the respirotropic strains in the intestine.

In the present study, virus distribution, local and systemic humoral immune response in SPF birds and the protection against lethal challenge conferred by vaccination with the VG/GA strain in commercial broiler chickens were evaluated and compared with the La Sota strain of NDV. In addition, complete nucleotide sequencing and full genome analysis of the VG/GA strain were performed to assess the genomic base of the strain phenotype.

Materials and Methods.

Viruses. The NDV La Sota strain used in the study was obtained from a commercial vaccine (Merial Select, Inc. Gainesville, Georgia, USA). The VG/GA/turkey/1987 strain used was the original isolate (embryo passage 3). Both strains were propagated by inoculation into embryonated fowl's eggs and titres of 10^8 median embryo infective dose/ml (EID_{50}/ml) were obtained for both viruses. The AF stock virus was kept at $-80^{\circ}C$ until use.

Experimental design. To test mucosal and systemic humoral immune response to vaccination, 90 1-day-old SPF chickens (Sunrise Farms, Catskill, New York, USA) were placed in groups of 30 in Biosecurity Level 2 isolation units where appropriated husbandry was provided. At 10 days of age, each group was inoculated by the oral/ocular route with 0.1 ml distilled water, or with a total dose of 10^7 EID_{50} in 0.1 ml VG/GA or La Sota strain, respectively.

Systemic humoral immune response. At 1, 4, 7, 11, 15 and 21 days post inoculation, eight birds in each group were bled and the serum samples used for enzyme-linked immunosorbent assay (ELISA) assessment using a commercial Newcastle disease antibody test (FlockCheck®; IDEXX, Maine, USA).

NDV-specific IgA detection. In each of the above-mentioned sampling days, four birds per group were selected to obtain tracheal and intestinal washes and bile. Briefly, chickens were killed humanely using a carbon dioxide chamber, the tracheas were clamped below the syrinx and 0.4 ml phosphate buffer saline flushed in and out 10 times. A similar procedure was applied to a 10 cm segment of the duodenum that was separated and clamped at one end. Bile was obtained by direct puncture of the gall bladder. Samples were placed in sterile containers and processed fresh. NDV-specific IgA levels in tracheal and intestinal lavages and bile were assayed by duplicate using an indirect ELISA as described previously (Raj & Jones, 1996), except that the coating antigen used was the La Sota strain of NDV and that the chicken IgA binding to the coating antigen was detected with a commercial goat anti-chicken IgA reagent (α-chain specific), conjugated with horseradish peroxidase (Bethyl Laboratories, Inc. Montgomery, USA). The optical density at 650 nm was measured using a Precision microplate reader (Molecular Devices. Inc., New York, USA). Corrected optical density values were calculated by subtracting the optical density values of non-antigen-coated wells from those of test wells.

Total IgA detection. To estimate the total (unspecific) IgA production in the respiratory and intestinal mucosa, a commercial ELISA chicken IgA quantification kit (Bethyl Laboratories, Inc., Montgomery, Texas, USA) was used on tracheal washings and bile samples following the manufacturer's recommendations. Checkerboard titrations established an optimal conjugate dilution of 1/10 000.

Viral tissue distribution. Tissue samples were collected from the trachea, lung, duodenum and caecal tonsils at 1, 4, 7, 11, 15 and 21 days post inoculation and kept frozen at $-80^{\circ}C$ until processing. After enzymatic digestion (proteinase K) of the individual samples, the tissue tropism of the strains was assessed by reverse-transcriptase polymerase chain reaction (RT-PCR) using the high pure RNA tissue kit (Roche Diagnostics Co., Indianapolis, Indiana, USA) for RNA extraction and the one-step RT-PCR Kit (Titan-Roche Diagnostics Co.) for amplification. NDV-specific primers and protocols were used as described elsewhere (Seal *et al.*, 1995). The amplification products were analysed by electrophoresis on a 1.5% w/v agarose gel stained with ethidium bromide (0.5 μg/ml).

VG/GA strain vaccine-challenge trial. A total of 288 1-day-old broiler chickens (Merial Select) with average maternal antibody ELISA titres of 1840, were randomly divided into nine groups of 32 birds each and vaccinated by eye drop at day 1 and/or day 14 with a total dose of 10^7 EID_{50} in 0.1 ml VG/GA and/or La Sota strain; one group remained as unvaccinated control. The treatment combinations are presented in Table 1. On days 14, 21, and 28 the NDV-specific ELISA test was performed on serum samples from eight birds per group. The IgA levels in trachea washings and bile were measured each sampling day in four birds per group as described above. On day 28 of the experiment, the birds were challenged by the intramuscular route with a dose of 10^4 EID_{50} per bird of the velogenic Texas GB strain of NDV.

VG/GA strain genome amplification. To assess the genomic basis of the tissue tropism of the VG/GA strain, a primer-sequence-independent amplification method was used on 200 l VG/GA stock allantoic fluid as described elsewhere (Djikeng *et al.*, 2006).

Sequence data, nucleotide sequencing and alignment analysis. All nucleotide sequencing reactions were performed with fluorescent dideoxynucleotide terminators in an automated ABI sequencer (ABI 3700 automated sequencer; Applied Biosystems Inc., Foster City, California, USA). Nucleotide sequence assembly and editing were conducted with the CodonCode sequence analysis software package. Comparison sequences were retrieved from GenBank public databases and used to generate alignments. Accession numbers for the 28 full genome NDV sequences used for comparison and alignment are as follows: AF375823, NC002617, AF309418, Y18898, AY845-400, AF077761, DQ60053, AY225110, AY562991, DQ097394, AY935499,AY935500, AY935498, AY935489, AY741404, AY562990, AY562986, AY562987, AJ880227, AY562989, AY562988, AY865652, AY562285, DQ659677, DQ485231, DQ485230, DQ485229, AF431744.

Alignments of complete genomes were performed using BioEdit v. 5.0.9 (Department of Microbiology, North Carolina State University, Raleigh, North Carolina, USA) with the ClustalW program followed by manual editing. Phylogenetic tree construction was done with Phyml V2.4.4 (for bootstrap analysis) under the General Time Reversible (GTR)

Table 1. *NDV vaccine-challenge trial local and systemic humoral immunity, and protection against lethal challenge*

Group	Age									
	14 days			21 days			28 days[c]			42 days
	ELISA[a] titres	Trachea[b] NDV IgA	Bile IgA	ELISA titres	Trachea NDV IgA	Bile NDV IgA	ELISA titres	Trachea NDV IgA	Bile NDV IgA	Challenge results: number of dead (% protection)
VG/GA day 1	134^{A}	0.45^{B}	0.93^{A}	403^{B}	1.02^{B}	1.16^{A}	1172^{B}	1.21^{B}	1.96^{A}	1/19 (95%)
VG/GA days 1 + 14	62^{A}	0.41^{B}	1.01^{A}	261^{C}	1.04^{B}	1.57^{A}	1865^{A}	1.18^{B}	2.41^{A}	0/18 (100%)
VG/GA day 14	25^{A}	0.05^{C}	0.02^{C}	47^{C}	0.19^{C}	0.31^{B}	952^{B}	0.90^{B}	1.40^{B}	0/20 (100%)
La Sota day 1	271^{A}	0.80^{A}	0.42^{B}	1049^{A}	1.33^{A}	0.42^{B}	1578^{A}	1.76^{A}	1.04^{B}	0/19 (100%)
La Sota days 1 + 14	209^{A}	0.72^{A}	0.39^{B}	1231^{A}	1.23^{A}	0.57^{B}	1913^{A}	1.98^{A}	1.27^{B}	0/19 (100%)
La Sota day 14	5^{A}	0.02^{C}	0.06^{C}	483^{C}	0.65^{B}	0.21^{C}	874^{B}	1.82^{A}	0.42^{C}	0/19 (100%)
VG/GA day 1 + La Sota day 14	92^{A}	0.40^{B}	1.01^{A}	437^{B}	0.72^{B}	1.81^{A}	1514^{A}	1.44^{B}	2.13^{A}	0/20 (100%)
La Sota day 1 + VG/GA day 14	216^{A}	0.74^{A}	0.48^{B}	547^{B}	1.44^{A}	1.74^{A}	1485^{A}	1.67^{A}	2.29^{A}	1/19 (95%)
Control challenged	24^{A}	0.05^{C}	0^{C}	22^{C}	0.07^{C}	0^{C}	42^{C}	0.05^{C}	0.1^{C}	9/9 (0%)

[a]Eight birds per group were tested and results are expressed as the geometric mean of the ELISA titres. [b]Four birds per group were sampled for local immunity; results are expressed as the average of the corrected optical densities. [c]Birds were challenged with a lethal dose of Texas GB strain at 28 days of age and observed for 14 days. The same uppercase capital letters within columns indicate no significant differences ($P < 0.05$).

model of nucleotide substitution with estimated proportions of invariable sites, ML base frequencies estimates, four substitution rate categories, and an affixed γ-distribution parameter. Coding regions were identified by translating the corresponding open reading frame with Bioedit, and protein alignments were performed with the ClustalW program.

Statistical analysis. All statistical analysis was performed using the Sigma Stat 3.0 software. Dunn's method and the Student Newman Test (SNT) were performed at $P \leq 0.05$.

Results

Serum IgG levels of VG/GA-vaccinated and La Sota-vaccinated SPF birds. The profile of the ELISA results obtained at the different sampling time points from the SPF birds vaccinated with VG/GA or La Sota strains of NDV corresponds with a primary progresive immune response regardless of the vaccine virus used (Figure 1). Both viruses induced IgG antibody levels that were statistically different ($P < 0.05$) from the unvaccinated control groups at days 11, 15 and 21 post vaccination. No statistical differences within the strains tested were observed.

NDV-specific IgA levels in biological samples of VG/GA-vaccinated and La Sota-vaccinated SPF birds. The IgA levels detected in the tracheal and intestinal washings and bile are shown in Figure 2a, b and c, respectively. The tracheal washings, representing the respiratory component of the mucosal immune response, showed that both viruses were able to induce measurable levels of NDV-specific IgA. The La Sota strain of NDV induced overall higher levels of IgA when compared with the VG/GA strain, but both groups differed ($P < 0.05$) from the unvaccinated controls. For the bile samples, higher IgA levels ($P < 0.05$) were observed in the birds vaccinated with the VG/GA strain of NDV. Similar results were observed for the intestinal washings.

Total IgA in biological samples of VG/GA-vaccinated and La Sota-vaccinated SPF birds. No significant differences ($P < 0.05$) were observed in the levels of total (unspecific)

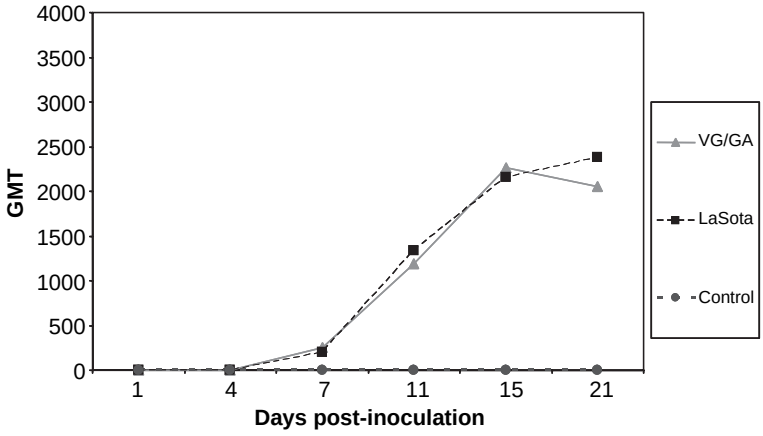

Figure 1. *Newcastle disease ELISA serology. Results are expressed as the geometric mean of the ELISA titres (GMT). No statistical differences were observed between the vaccine strains. Both differed* P *<0.05 from the non-vaccinated control.*

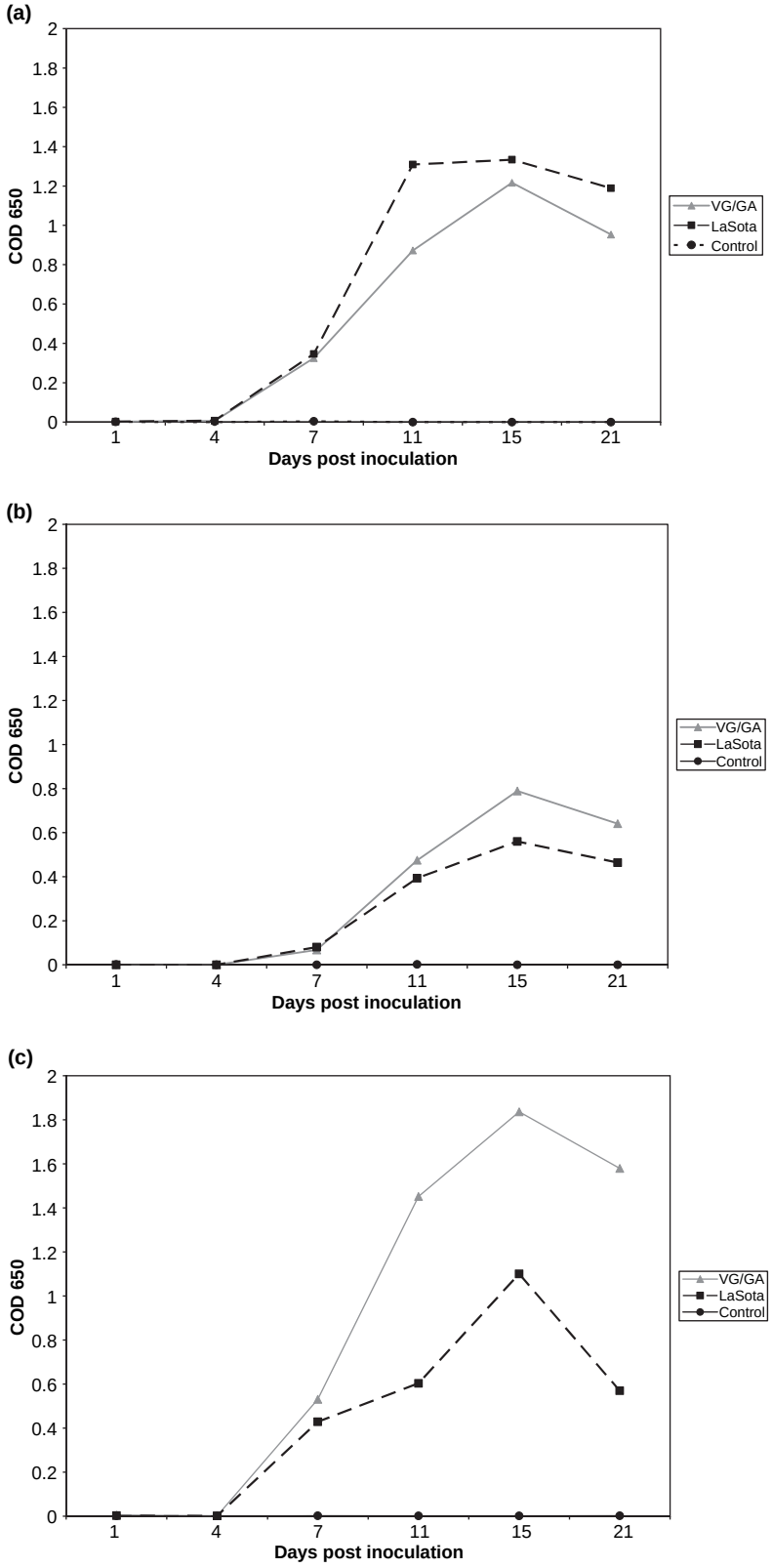

Figure 2. *Mucosal immunity measured as IgA levels in SPF birds. 2a: IgA levels in tracheal washings. 2b: IgA levels in intestinal washings. 2c: IgA levels in bile. Results are expressed as the corrected optical density at 650 nm (COD650).*

IgA between the control and the vaccinated groups in all of the samples tested (data not shown).

RT-PCR detection of NDV in respiratory and intestinal tissues of SPF birds. To confirm the ability of VG/GA strain to replicate in the respiratory and intestinal tract of chickens, the virus distribution of the VG/GA was assessed by RT-PCR in SPF chickens vaccinated at 10 days of age and compared with the tissue tropism of La Sota strain. Results are presented in Table 2. The VG/GA strain was detected both in the respiratory and in the intestinal tract, with a preferential tropism for the later. The presence of the VG/GA in the trachea and lungs was transient and detectable only at 4 and 7 days post

Table 2. *RT-PCR detection of virus tissue distribution*

RT-PCR treatment	Organ																							
	1 day post inoculation				4 days post inoculation				7 days post inoculation				11 days post inoculation				15 days post inoculation				21 days post inoculation			
	T	L	D	Ct	T	L	D	Ct	T	L	D	Ct	T	L	D	Ct	T	L	D	Ct	T	L	D	Ct
VG/GA	−	−	+	−	+	+	+	+	+	+	+	+	−	−	+	+	−	−	−	+	−	−	−	−
La Sota	+	−	−	−	+	+	−	−	+	+	−	−	+	+	−	−	−	−	−	−	−	−	−	−
Control	All tissues negative																							

Samples from four birds where obtained and pooled each day. T, trachea; L, lung; D, duodenum; Ct, caecal tonsil; +, RT-PCR-positive signal (250 base pair amplicon); −, no amplification.

inoculation. As early as 24 h after infection, the VG/GA nucleic acids were detected in the duodenum and remained detectable until day 11, in the caecal tonsils the RNA was amplified from 4 to 15 days post inoculation. The RT-PCR confirmed the respirotropic nature of La Sota strain by amplifying the virus from day 1 up to day 11 in the respiratory tissues.

Vaccine challenge trial. The different vaccination schedules assessed resulted in protection against lethal challenge; all the unvaccinated challenged controls died within the observation period. The results for the experimental vaccine-challenge trial and the immunoglobulin measurements are summarized in Table 1. It was demonstrated that, despite the presence of the maternal antibodies, early vaccination with the VG/GA in broilers generates systemic and mucosal NDV-specific antibodies accompanied by protection levels between 95% and 100% against a lethal velogenic challenge. The VG/GA efficacy was equivalent to the protection offered by the La Sota strain. Combinations of the two strains were also highly efficacious. Systemic immune response varied among groups. Regardless of the vaccine strain administered first, the treatments with two doses induced higher antibody titres than those where only one vaccine was applied. All the vaccinated groups differed significantly ($P < 0.05$) from the unvaccinated controls at days 21 and 28 post inoculation. The results for the IgA levels in respiratory and intestinal tract in the broiler chickens were equivalent to those observed in the SPF birds: higher IgA levels in the trachea washings for the La Sota strain and higher IgA levels in the intestinal tract of the VG/GA vaccinated birds.

Complete nucleotide sequence of VG/GA strain. Phylogenetic comparison of the VG/GA genome with 28 NDV full genome sequences available in GenBank is shown in Figure 3. The analysis based on the full genome sequences indicates that the VG/GA strain can be grouped within the class II, genotype II, which corresponds to most of the respirotropic vaccine strains used in the poultry industry and differed from the other lentogenic strains with enteric

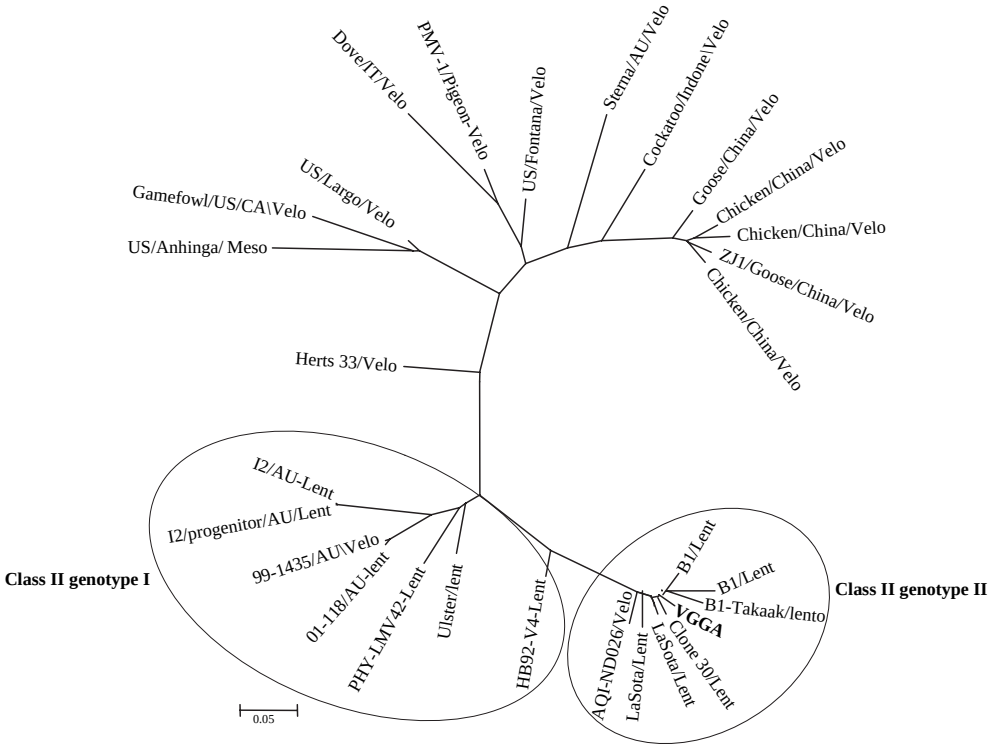

Figure 3. *Full nucleotide phylogeny of the VG/GA strain of NDV. The VG/GA strain grouped among the class II genotype II viruses, which corresponds with most of the classic poultry vaccine strains.*

tropism (QV4, Ulster, PHY-LMV42) that belong to the class II in the genotype I.

Differences were observed at both the nucleotide and amino acid levels when the genes and proteins of strains VG/GA and La Sota were compared. A total of 66 residue substitutions were distributed in all the six structural proteins analysed. Four amino acid changes where observed between the VG/GA and La Sota F proteins. The first of these, V-106-M, was located near the heptad repeat 4 domain in the F_2 polypeptide. The second and third were contiguous changes (I-135-M and T-136-A) located at the end of the 20 extensively hydrophobic N-terminal residues of F_1.

The differences among the strains were located in the globular head of the HN protein and included substitutions of hydrophobic residues with polar residues; for instance, a small non-polar glycine at position 169 in La Sota is substituted by a large polar arginine residue in the VG/GA strain HN protein, and a large non-polar valine is substituted by a small polar glutamate. The length of the HN protein of the VG/GA strain was 577 amino acids.

The nucleotide sequence comparison of the VG/GA strain L gene revealed a single nucleotide insertion at position 3870. This mutation is compensated by a nucleotide deletion downstream at position 3958, which results in a 30-amino-acid substitution in domain V of the L protein. The mutation is present in the VG/GA and absent in the La Sota strain. At least five amino acid substitutions were observed in each of the remaining proteins: matrix, nucleocapsid and phosphoprotein. All differences are summarized in Table 3.

Discussion

The RT-PCR results demonstrate that the VG/GA strain can be detected both in the respiratory and intestinal tract of chickens, while the La Sota strain was detected in the respiratory tract. Although we did not perform virus isolation from the different tissue samples, a very high positive correlation between virus isolation and RT-PCR detection that validates the use RT-PCR for NDV tissue tropism studies has been reported (Gohm *et al.*, 2000; Ganapathy *et al.*, 2005; Wakamatsu *et al.*, 2007). An association between the site of replication and the levels of IgA production was observed and can be explained by the virus replication and host antigen recognition mechanisms proposed for NDV (Peeters *et al.*, 1999; Alexander, 2001; de Leeuw *et al.*, 2005). Active replication in the mucosa induces virus protein production and local antigen presentation through MHC class I and II molecules stimulating a T-dependent B-cell response at the site of infection in the form of

Table 3. *Amino acid changes among VG/GA and La Sota strains of NDV.*

Protein	Amino acid La Sota	Residue position	Amino acid VG/GA
HN	G	169	R
	Y	203	H
	S	324	T
	V	495	E
	T	522	I
F	V	106	M
	I	135	M
	T	136	A
	V	255	I
M	G	29	D
	L	48	S
	I	104	V
	I	114	M
	V	196	I
N	M	389	R
	K	390	R
	D	401	E
	D	402	V
	I	407	D
P	E	8	D
	F	109	L
	P	164	L
	T	317	I
	K	352	R
L	P	18	S
	Q	97	E
	M	187	I
	K	191	R
	T	253	M
	T	305	I
	S	897	P
L (V domain)	CHLTFTYPMILKGCSLK KESKRGMWFTNRV	1287 to 1316	VSPYIHISNDSQRLFTEEGVK EGNVVYQQI
	V	1366	A
	S	1385	L
	E	1620	G
	I	2103	K

IgA-producing plasma cells in the intestine (Al-Garib *et al.*, 2003).

The importance of local antibodies in the defence mechanism against viral infection has been emphasized in recent years (Scott, 2004). It appears that local immunity acts as a barrier at surfaces where primary viral infections occur, thereby interfering with further spread of the virus (Jayawardane & Spradbrow, 1995; Russell, 1993). Birds have a well-developed mucosal immune system; its characteristics include local production and secretion of IgA antibodies and traffic of IgA-producing plasma cells (Zigterman *et al.*, 1993; Jayawardane & Spradbrow, 1995; Al-Garib *et al.*, 2003). The IgA class predominates and is detectable in tears, saliva, tracheal and intestinal washes and bile. In this trial, the mucosal immune response measured as the levels of IgA induced by VG/GA strain vaccination was assessed. The replication pattern of VG/GA strain induced a stronger localized mucosal immune response in the intestinal tract shown by an increased production of NDV-specific IgA. This feature may represent a competitive advantage in the event of a velogenic viscerotropic challenge where the virus has been reported to induce massive destruction of intestinal lymphoid areas and extensive ulceration of overlying intestinal epithelium associated with active viral replication (Brown *et al.*, 1999).

No significant differences were observed in the levels of total (unspecific) IgA between the unvaccinated control and the vaccinated groups, suggesting that there is no measurable effect of NDV vaccination in the overall IgA load of the mucosal tissue in chickens. This observation could be explained as the consequence of commensal and/or pathogenic colonization of the respiratory and intestinal epithelial surfaces that represent the putative site of initial antigen encounter (Brandtzaeg, 2003). Furthermore, epithelial cells have been proved to provide co-stimulatory signals promoting terminal differentiation of B cells oriented towards IgA production, generating relatively high and constant levels of the immunoglobulin (Brandtzaeg, 2003; Scott, 2004).

Systemic humoral immunity represented by neutralizing IgG antibodies against NDV HN and F glycoproteins is a relevant component of the bird's protection against infection. Antibody-based virus neutralization, complement activation and immune complex formation pathways are important for the control of NDV and correlate positively with protection (Seal *et al.*, 2000). The trend observed in the serum IgG levels for the VG/GA and La Sota viruses corresponds with a primary immune response with a progressive time-dependent increase of the antibody titres.

Despite the presence of the maternal antibodies in broilers, early vaccination with the VG/GA strain afforded 95% to 100% protection against lethal challenge, equivalent to the protection offered by the La Sota strain. These results validate anecdotal data obtained from field observations and confirm results from vaccine-challenge trials performed in SPF chickens (Beard *et al.*, 1993) and in quails (Silva *et al.*, 2004). The efficacy of the different treatment combinations using both VG/GA and La Sota strains, demonstrated the feasibility of using a multiple strain vaccine protocol with VG/GA strain for initial vaccination when high challenge is present and field revaccination is scheduled.

Based on how fast the VG/GA is cleared from the respiratory tract after priming the mucosal immune response, the mucosal IgA production both in the respiratory and the intestinal tract and the levels of protection afforded by single or multiple doses of the vaccine, initial vaccination with the VG/GA strain may be advantageous for the integrity of the respiratory mucosa of young chickens when multiple vaccination and field exposure is expected. Previous reports on morphometric analysis and comparison of tracheal thickness after vaccination with different vaccine strains indicated that La Sota and Ulster strains had equivalent virulence and both caused higher swelling of tracheal mucosa than VG/GA strain (Nunes *et al.*, 2002).

Antigenic (Alexander *et al.*, 1998) and genetic diversity (Aldous *et al.*, 2003) are recognized within the NDV isolates, which are all members of the APMV-1 serotype. Based on the nucleotide sequence there have been at least six distinct lineages identified for NDV (Aldous *et al.,* 2003). A more traditional classification using the full-length sequence has been reviewed and comprises two major divisions represented by class I and class II, with class II being further divided into nine genotypes (Czegledi *et al.*, 2006). For instance, the US isolates of NDV identified in the 1940s and most of the respirotropic vaccines used today to control Newcastle disease are class II, genotype II. After full genome sequence analysis, the VG/GA strain was grouped within the class II, genotype II. These results are in agreement with previous reports based on partial sequences (Seal *et al.*, 1995; Aldous *et al.*, 2003). The VG/GA phylogeny differed from the other vaccine strains able to replicate in the intestine; these strains belong to class II, but genotype I. The Ulster, QV4 derivates, and PHY-LMV42 strains are more closely related than VG/GA to the Australian isolates for which drift mutations at the cleavage site responsible for increases in virulence has been reported. Over time, some viruses in this group have acquired more basic amino acids and the leucine (L) change to phenylalanine (F) at the F_0 cleavage site, which made the final Australian virus highly virulent (Gould *et al.*, 2001).

The nucleotide and amino acid composition of the VG/GA strain were compared with the La Sota strain; differences were observed at both levels. The F glycoprotein of NDV is a type I integral membrane protein that has been shown to be involved in virus penetration and cell fusion (Morrison, 2003). The amino acid changes between the F proteins of the VG/GA and La Sota strains included contiguous substitutions in the fusion peptide, which is conserved among paramyxovirus (up to 90% identity) and is directly involved in fusion promotion (Horvath & Lamb, 1992). The mutations observed in the fusion peptide of the VG/GA strain F protein are unique and may be associated with its phenotype.

The nucleotide and amino acid differences between the VG/GA and La Sota HN protein were located in the globular head of the HN that has been proposed to be a dynamic molecule that switches from one conformational state to another, resulting in a change of an active site that is responsible for both receptor binding and neuraminidase activity (de Leeuw *et al.*, 2005). Amino acid substitutions at specific locations can have a profound effect on the folding and function of the proteins and could be responsible for the differential

tropism of these two strains. Romer-Oberdofer *et al.* (2003) indicated that the length of the HN protein may play a role in the ability of the virus to spread and propagate in various organs after inoculation. The 577 amino acids of the VG/GA HN protein is also the length of the La Sota HN protein and has been reported for both virulent and avirulent strains, which may disregard the role of the protein size in the tissue tropism of the VG/GA strain.

The mutation in the domain V of the L protein present in the VG/GA has been documented previously; the authors reported the existence of two forms of the L protein of NDV after sequence analysis of NDV isolates from different backgrounds (Kusumaningtyas *et al.*, 2004). The association of this genotype with virus replication and tissue tropism is yet to be determined for NDV; however, domain V has been proposed to play an important role in transcription and thermosensitivity of isolates of vesicular stomatitis virus and Sendai virus (Banerjee, 1987; Cortese *et al.*, 2000). An interesting observation is that the clone 30 strain, which is a more attenuated from of La Sota strain of NDV (lower post-vaccine reactions), has the same amino acid profile as the VG/GA strain in this segment of the L protein (Romer-Oberdorfer *et al.*, 1999). The changes observed in proteins associated with tissue tropism may explain the differential phenotype of the VG/GA strain; however, further studies including the generation of a reverse genetic system and nucleotide substitution studies are required to verify the significance of these changes.

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